

## Epidemiologic and Clinical Features of Cutaneous Leishmaniasis in Southeastern Tunisia

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**Abstract.** Species-specific diagnosis was performed in 66 patients with cutaneous leishmaniasis (CL) living in Tataouine focus in southeastern Tunisia. *Leishmania* DNA was extracted directly from dermal scrapings ( $n = 66$ ) and from parasites obtained in culture ( $n = 12$ ). Species were identified by using polymerase chain reaction–restriction fragment length polymorphism analysis for internal transcribed spacer region 1 and isoenzyme analysis. *Leishmania tropica* and *L. major* were identified in 31 (47%) and 35 (53%) cases respectively. *Leishmania tropica* CL cases were geographically scattered, and *L. major* CL cases were clustered. Lesions caused by *L. tropica* were mostly single (83.8%) and face-localized (55.8%), and lesions caused by *L. major* were multiple (57.1%;  $P < 0.001$ ) and situated on limbs (83.7%;  $P < 0.001$ ). For both species, most lesion onsets were reported during June–January. However, lesions that emerged during February–May were mainly caused by *L. tropica* (83.3%;  $P < 0.01$ ). Moreover, the delay before seeking medical advice was higher for *L. tropica* infections than for *L. major* infections ( $P < 0.05$ ).

### INTRODUCTION

Cutaneous leishmaniasis (CL), which is caused by *Leishmania tropica*, is endemic in the southeastern Tunisia. It occurs as scarce cases in microfoci located in the arid mountains of Tataouine. Among zymodemes attributable to the polymorphic *L. tropica* complex, MON-8 was found to be endemic in this region and belonging to a separate species (*L. killicki*).<sup>1</sup> Recently, all 35 *L. tropica* zymodemes, the *L. killicki* zymodeme, and a new zymodeme related to *L. killicki* (MON-301 isolated in Algeria) have been considered as belonging to a single broad *L. tropica* complex. In this complex of four clusters, cluster A (including *L. killicki* parasites) is the most distant from the others.<sup>2</sup>

Since its first description, there has been no epidemiologic investigation of the CL focus in Tataouine.<sup>1</sup> Only sporadic isoenzyme typing of *Leishmania* strains from patients in this region was conducted.<sup>3,4</sup> These studies confirmed the presence of *L. tropica* MON-8 and identified concomitant cases of CL caused by *L. major*. However, the number of reported identifications on the basis of isoenzyme typing was too small to assess the respective prevalence of the two forms of CL and study their epidemiologic features in this region. Therefore, efforts to provide species-specific leishmaniasis diagnosis at the primary healthcare level are of utmost importance to gain a better knowledge of disease epidemiology and to develop efficient control measures. Furthermore, the ability to distinguish between *Leishmania* species is also crucial for evaluating clinical prognosis and adjusting therapeutic approaches. Compared with cases caused by *L. major*, those caused by *L. tropica* may persist longer and may be less responsive to treatment.<sup>5</sup>

In leishmaniasis-endemic areas, diagnosis of CL is essentially based on detection of *Leishmania* amastigotes in Giemsa-stained smears. However, microscopic examination does enable identification of *Leishmania* species.<sup>6</sup> Culture methods are more helpful in species characterization, but require laboratory facilities, technical expertise, and are dif-

ficult to carry out at the primary healthcare level.<sup>3,4,6</sup> During the past decade, molecular tools for diagnosis of CL had been extensively used.<sup>6</sup> Essentially based on polymerase chain reaction (PCR) technology, these methods are particularly useful for identification of *Leishmania* species directly on clinical samples without the need for prior culture.<sup>6,7</sup> Moreover, simplified methods of specimen collection and transport have led to a significant improvement in individual and epidemiologic molecular diagnosis of CL.<sup>6,8</sup>

The purpose of this study was to identify *Leishmania* species that cause CL in southeastern Tunisia and to define the epidemiologic and clinical features of the disease in this region.

### MATERIALS AND METHODS

**Study area.** The study area corresponds to the historical focus of *L. tropica* MON-8 described by Rioux and others in 1986.<sup>1</sup> Situated in the northeastern part of the Sahara Desert, this region covers four districts of the Tataouine governorate, which is located in a mountainous area with a moderate altitude (300 meters) (Figure 1). It has a population of 115,750 persons living in towns and villages that are built on mountain flanks and that have an arid climate (Figure 1). The regional landscape is characterized by poor vegetation and rocky escarpment. During 1999–2008, an average of 143 CL cases/year (range = 46–291 cases) was recorded (data were obtained from the regional directory of health).

**Patients.** The study was performed in the primary healthcare unit of Tataouine, which is the regional reference laboratory for microscopic *Leishmania* identification in the governorate. During October 2008–September 2009, 177 patients from the study area with a clinical presentation suggestive of CL were referred to this unit for laboratory diagnosis. One hundred twenty patients were enrolled in this study after informed consent was obtained from the patients (or their parents if children). An additional sample was obtained to identify *Leishmania* species. Data concerning age, address, and travel history were obtained from each patient by using a standardized questionnaire. Date of onset of disease and number and location of the lesions were also obtained. The study protocol was reviewed and approved by the Regional Directory of Health of Tataouine, the institutional authority in the study area.

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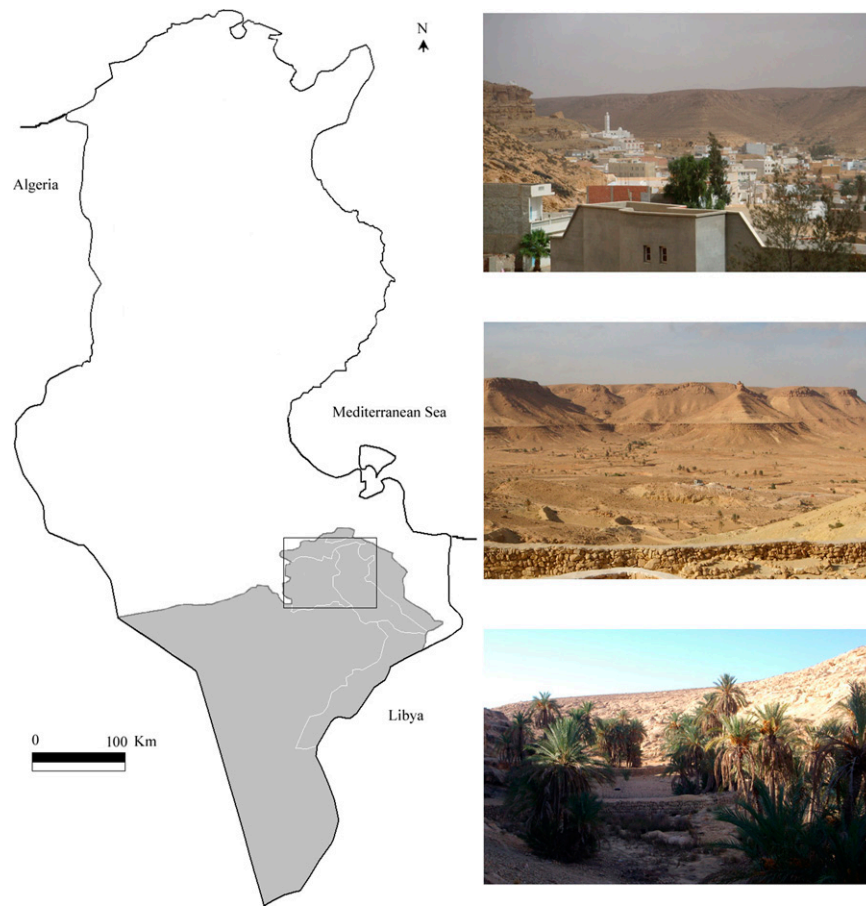


FIGURE 1. *Leishmania tropica* focus in southeastern Tunisia. The map on the right shows the governorate of Tataouine (shaded black). The study area is situated in the northeastern part of the governorate. The photographs on the left show the regional landscape. The photograph on the top left shows the town of Ghomrassen. This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

**Sample collection.** A 2–3 mm superficial incision was made on the inflammatory border of the lesion by using a sterile lancet. For conventional diagnosis, a dermal scraping was obtained by using the same lancet to prepare a slide smear for microscopic examination. For molecular analysis, additional dermal scrapings were obtained from 120 patients by using a sterile cytology brush (Deltalab, Barcelona, Spain) designed to enable gentle cell sampling and collection of uterine endocervical samples. The skin-brushed material was eluted in 200  $\mu$ L of phosphate-buffered saline, stored in plastic tubes at 4°C, and transported within one week to the Department of Parasitology at Pasteur Institute in Tunis for PCR analysis. An aspirate from the same lesion from 27 patients was obtained by using a Pasteur pipette and cultured on Novy-MacNeal-Nicolle medium.<sup>9</sup>

**Standard diagnostic procedures.** Smears on glass slides were fixed with methanol, stained with Giemsa, and examined at the regional level. Cultures were transported to the Department of Parasitology of the Pasteur Institute in Tunis, incubated at 26°C, and evaluated microscopically every six days.<sup>8</sup> Positive cultures were subsequently transported to the Pasteur Institute of Algiers in Algeria for isoenzyme typing by using the procedure of Rioux and others.<sup>10</sup>

**DNA extraction and PCR analysis.** DNA was extracted by using the Qiamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and performed directly on dermal scraping diluted in phosphate-

buffered saline or on parasites obtained in culture. Primers LITSR and L5.8S were used for amplification of the ribosomal internal transcribed spacer 1 (ITS1) region, which separates genes coding for small subunit ribosomal RNA and 5.8 S ribosomal RNA.<sup>7</sup> The PCR products were digested with *Hae*III (Fermentas, Glen Burnie, MD) at 37°C for 1 hour. Restriction profiles were analyzed by electrophoresis on 3% agarose gels and compared with those described by Schonian and others.<sup>7</sup>

**Statistical analysis.** Statistical analysis was conducted by using SPSS version 13.0 (SPSS, Inc., Chicago, IL). Quantitative variables are reported as mean and SD or median and interquartile range (IQR) if data did not show a normal distribution. The Pearson chi-square test was used to compare binary qualitative variables (degrees of freedom = 1), and the Student *t*-test and Mann-Whitney non-parametric U test were used to compare quantitative variables. A test result was considered significant if the *P* value was < 0.05.

## RESULTS

Overall, CL diagnosis was confirmed in 102 of 177 suspected cases by microscopic examination and/or ITS1-PCR. *Leishmania* amastigotes were identified in 96 of 177 skin smears (54.2%). *Leishmania* DNA was detected in 70 of 120 samples examined by ITS1-PCR (58.3%). Only 64 of 70 samples amplified by PCR were also positive by microscopic

examination. However, microscopic analysis of 50 samples that were negative by PCR assay did not detect *Leishmania*.

*Leishmania* promastigotes were identified in 16 of 27 cultivated samples (59.2%). These 16 samples were also positive by microscopy and PCR. Twelve *Leishmania* strains isolated from positive cultures were subjected to isoenzyme and molecular typing. *Leishmania tropica* MON-8 and *L. major* MON-25 were identified in 4 (33.3%) and 8 (66.7%) cases, respectively. Molecular and isoenzyme typing results were concordant in all cases.

Using a PCR–restriction fragment length polymorphism (RFLP) assay, we amplified products obtained from dermal scrapings and identified the species in 66 samples. The RFLP band patterns of *L. tropica* (50 kD and 190 kD) and *L. major* (127 kD and 220 kD) were similar to those described

by Schonian and others.<sup>7</sup> *Leishmania tropica* and *L. major* were identified in 31 (47%) and 35 (53%) cases, respectively. Restriction profiles for either amastigotes from skin samples or promastigotes from cultures were similar.

Fifty males and 52 females in different age groups (mean  $\pm$  SD age = 30.20  $\pm$  21.3 years, range = 9 months to 82 years) had cutaneous leishmaniasis. No statistically significant differences were observed in ages of infected patients with *Leishmania* species (mean  $\pm$  SD age = 33.97  $\pm$  23.72 years for the *L. major* group versus 26.32  $\pm$  18.06 years for the *L. tropica* group;  $P = 0.156$ ).

Cases of CL caused by *L. tropica* were geographically scattered (infected patients lived in more than 10 villages) and cases of CL caused by *L. major* were clustered (65% of infected patients lived in marginal neighborhoods of Ghomrassen) (Figure 2).

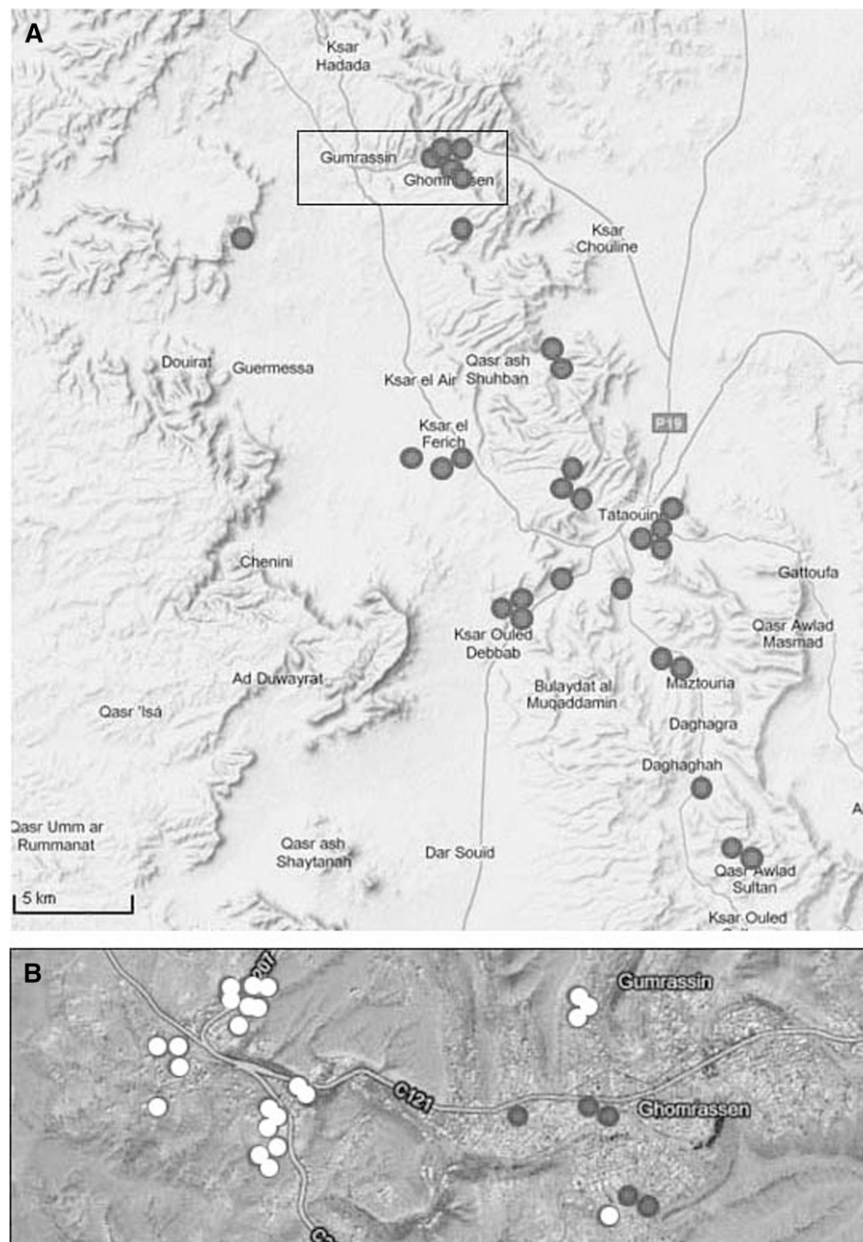


FIGURE 2. Distribution of cutaneous leishmaniasis in Tunisia according to *Leishmania* species. **A**, geographic distribution of cutaneous leishmaniasis cases caused by *L. tropica* in the study area (gray circles). **B**, geographic distribution of *L. tropica* (gray circles) and *L. major* (white circles) in the town of Ghomrassen.



TABLE 1

Number of cases of cutaneous leishmaniasis with single and multiple lesions and site of lesions according to species, Tunisia\*

Characteristic	Single lesions			Multiple lesions					Total
	F	A/H	Ft	F	A/H	Ft	F plus A/H	A/H plus Ft	
<i>L. major</i>	2	9	4	2	5	7	2	4	35
<i>L. tropica</i>	15	11	0	1	0	0	3	1†	31
Total	17	20	4	3	5	7	5	5	66

\* F = face; A/H = arm/hand; Ft = foot.

† Species identification from an arm lesion.

*Leishmania tropica* CL lesions were mostly single (83.8%) and located on the face (55.8%), and *L. major* CL lesions were mainly multiple (57.1%) ( $\chi^2 = 11.75$ ,  $P = 0.0006$ ) and situated on the limbs (83.7%) ( $\chi^2 = 12.22$ ,  $P = 0.0004$ ). Hands were infected by both species and feet were infected only by *L. major* (Table 1).

Most lesions (81%) were reported during June–January and only 19% were reported during February–May. Peaks of emerging cases were observed in August and December for *L. tropica* and in September and December for *L. major* (Figure 3). Lesions that emerged during June–January were caused mostly by *L. major* (64.7%), and lesions that emerged during February–May were caused mainly by *L. tropica* (83.3%) ( $\chi^2 = 9.08$ ,  $P = 0.002$ ).

All patients infected by *L. major* sought medical advice during the four months after lesion onset, and 84% of those infected by *L. tropica* consulted their doctors during the same period. Two patients infected with *L. tropica* had lesions evolving for more than one year at the time of diagnosis. Conversely, the diagnosis lag was higher for *L. tropica* (median = 60 days, IQR = 40–120 days) than for *L. major* (median = 60 days, IQR = 30–60 days) ( $P = 0.02$ ) (Figure 4).

## DISCUSSION

A variety of specimens, such as skin punch or ellipse skin biopsy, fine needle aspiration material, or dermal scraping,

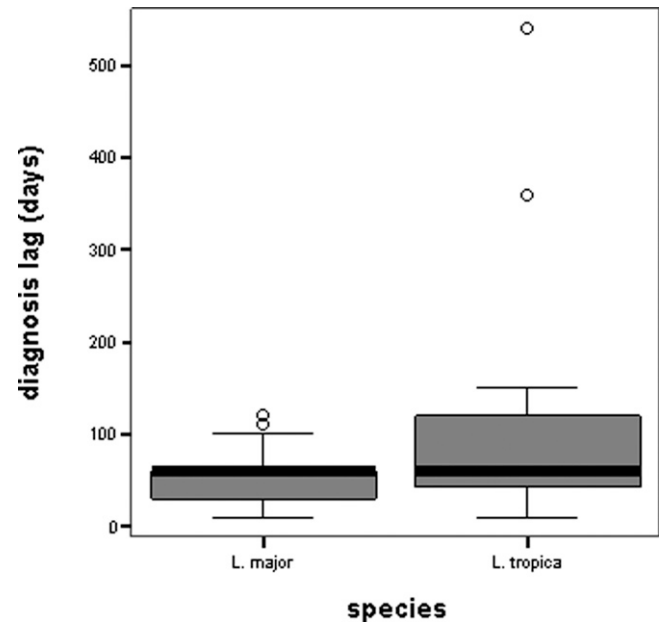


FIGURE 4. Diagnosis lag according to species for case of cutaneous leishmaniasis, Tunisia. The box plots show medians and interquartile ranges.

can be successfully used in CL diagnosis.<sup>6</sup> Compared with other collection techniques, dermal scraping enables simple sampling at the ambulatory level by personnel with minimum training.<sup>8</sup> It is commonly used in Tunisia, especially in leishmaniasis-endemic foci, where most cases are diagnosed at the regional level. The cytology brush used to collect additional dermal scrapings for PCR minimized the traumatic impact of the lancet, especially for lesions on the face and for children. It was simple and easy to use at primary health-care level. Convenient conservation conditions of samples enabled transport at 4°C to the reference laboratory for PCR testing.

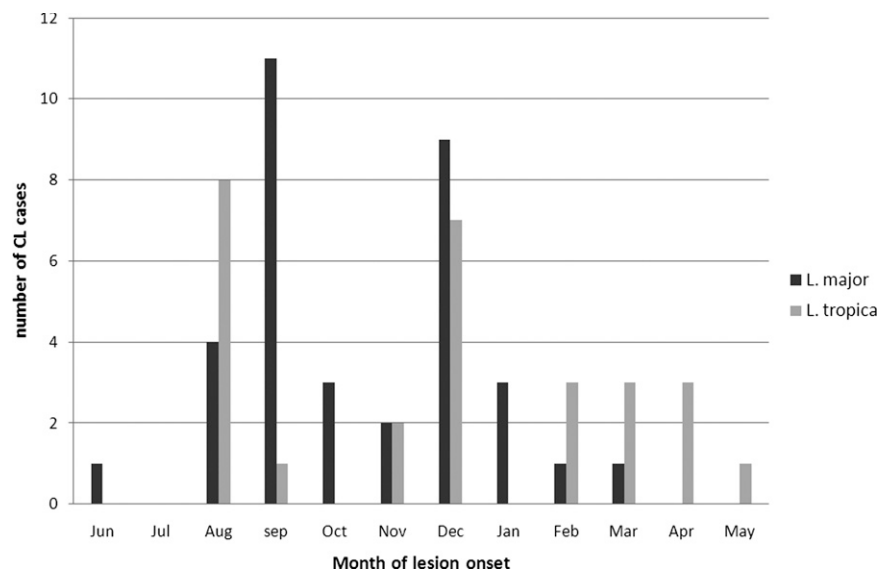


FIGURE 3. Number of cutaneous leishmaniasis cases according to the onset date of lesions (June 2008–May 2009) and species-specific diagnosis, Tunisia.

Microscopic examination of Giemsa-stained slides and promastigotes culture in Novy-MacNeal-Nicolle medium were the conventional techniques used. Laboratory diagnosis of CL was made by these methods in 54.2% and 59.2% of cases, respectively. The decreased sensitivity of these techniques has been reported.<sup>6</sup> It can be explained by the presence of a considerable number of CL cases with a chronic lesion or with a low parasitic load in skin tissue.<sup>6</sup>

Among all samples positive by conventional or molecular tools, the ITS1-PCR showed 100% sensitivity, which was higher than that of conventional methods (94.1%). Moreover, the ITS1 PCR-RFLP enabled easy species identification in 94% of PCR-positive cases with only one restriction enzyme (*Hae*III) for amplicon digestion. ITS1 showed have good sensitivity and the PCR-RFLP with *Hae*III was an efficient technique for *Leishmania* species identification.<sup>7</sup>

In the Tataouine region, *L. major* and *L. tropica* are currently the causative agents of CL. As reported, CL caused by *L. tropica* seems to be hypoendemic. It is found in communities in villages built on the flank of rocky mountains.<sup>1</sup> Cases of CL caused by *L. major* probably emerged in this area as a result of recent establishment of a high density of susceptible human populations at the margin of some villages. In this area, environmental conditions and development of agricultural activities may be favorable for transmission of *L. major*. Clustering of infections within these settlements may indicate spread and adaptation of transmission cycles to a peridomestic environment.<sup>11</sup> As described in other regions in Tunisia, exposure to *L. major* infection resulted in epidemics with a seasonal occurrence of cases.<sup>12</sup> For both species, reported human CL cases during June–January may suggest a long season of transmission in southeastern Tunisia. The two peaks of emerging cases in August–September and December are probably related to the seasonal activity of the respective phlebotomine sand fly vectors.<sup>13</sup> The different seasonal distribution patterns of *L. major* and *L. tropica* cases, observed during February–May is probably caused by specific variation in the incubation period.<sup>14</sup>

Cutaneous leishmaniasis caused by *L. major* and *L. tropica* differs not only in its epidemiologic features, but also in its respective clinical presentation and course of infection. Although lesions caused by *L. major* were typically multiple on the hands and feet, lesions caused by *L. tropica* were mainly single and on the face or hands.<sup>15</sup> Localization of lesions is probably associated with different biting behaviors of sand fly species involved in the transmission (*Phlebotomus papatasi* for *L. major* and *Paraphlebotomus* species, probably *P. sergenti*, for *L. tropica*).<sup>13</sup> Conversely, infection caused by *L. tropica* seems to be more insidious compared with *L. major* infection, with a longer incubation period and probably fewer inflammatory lesions. This finding could explain the long delay before infected persons seeking advice. Two patients infected by *L. tropica* had lesions lasting more than one year. This finding confirms the chronic tendency of this form of CL.<sup>1,15</sup> These clinical differences between *L. major* and *L. tropica* are consistent with those reported from the Middle East and central Asia.<sup>16</sup>

Our results highlight the possibility of introducing at primary healthcare level simple species identification tools. These tools will be useful in acquiring better knowledge of the epidemiology and management of CL cases,

especially in areas where more than one *Leishmania* species is transmitted.

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